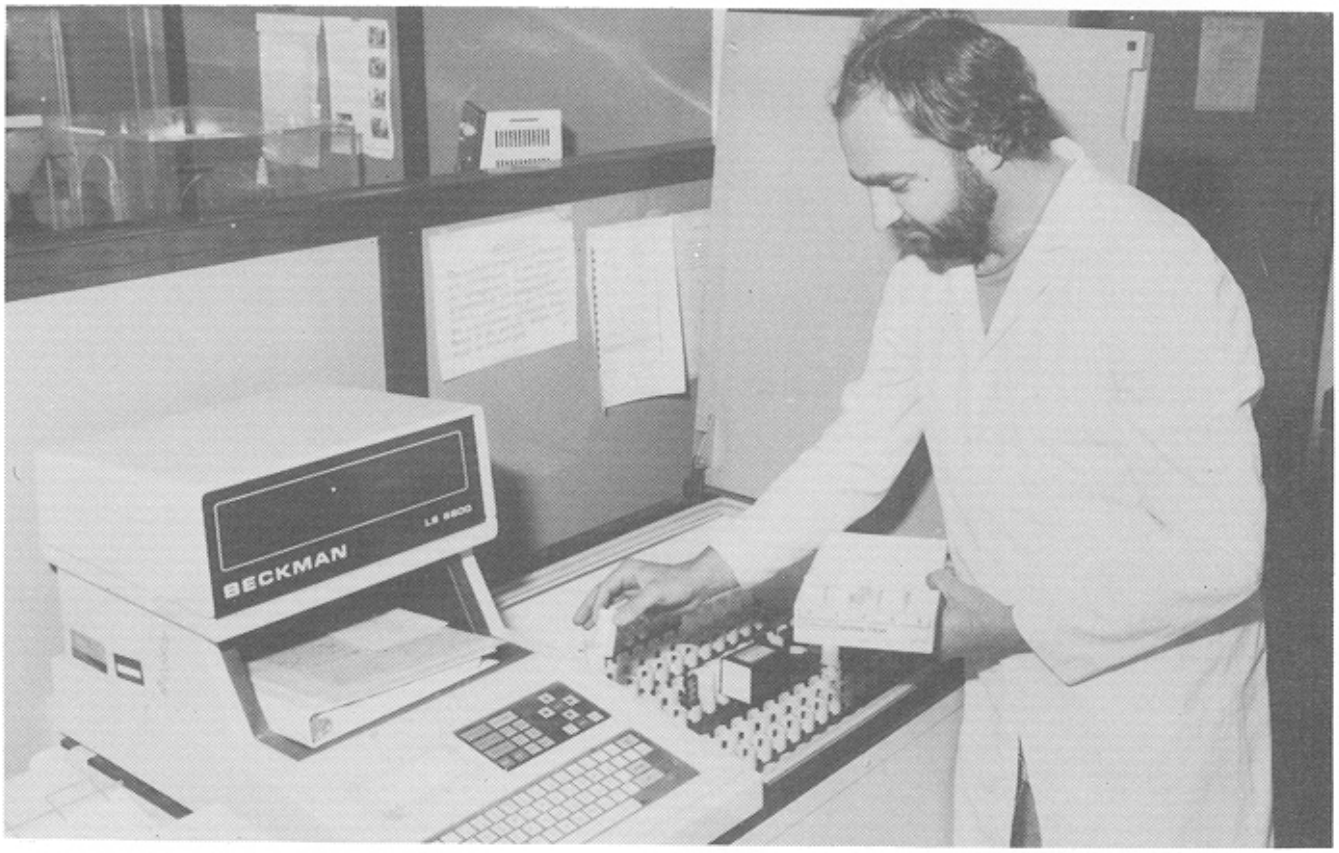


Dr. Jon Wilkes places into a scintillation counter vials holding different populations of trypanosome parasites that have been incubated with the anti-trypanosome drug Samorin®. The drug has been labelled with radioactive molecules. The scintillation counter measures the amount of radioactivity in each vial. This tells the scientist how much drug has been taken up by the different parasite populations. Drug-resistant populations have evolved ways of reducing their drug uptake.

RAPD fingerprinting of trypanosomes

CHARACTERIZING and diagnosing trypanosome infections in animals is complicated by the existence of many disease-causing subgenera, species and subspecies of the genus *Trypanosoma,* in addition to the occurrence of several apparently harmless trypanosome populations. Three subgenera—*Nannomonas, Duttonella* and *Trypanozoon*—cause large, widespread losses in livestock productivity throughout the tropical and subtropical regions of the world. Members of the Nannomonas subgenus cause the greatest ruminant losses due to trypanosomiasis in sub-Saharan Africa; five genetic types of parasites have been identified within this subgenus. Two subspecies in the *Trypanozooon subgenus—Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gumbiense*—cause life-threatening human diseases. Complicating the picture still further are mixtures of parasites of the same or different subgenera, species or subspecies infecting an animal simultaneously.

What is needed by both scientists and animal disease control workers are systems for quickly and efficiently distinguishing among the many trypanosome populations. Such systems would enable workers to make accurate diagnoses, to obtain sound epidemiological data and to optimize the use of chemotherapy to prevent and treat trypanosomiasis.

SEVERAL TECHNIQUES are used to distinguish the different populations of trypanosomes that infect people and their domestic animals. Antigen-trapping enzyme-linked immunosorbent assays (antigen-ELISAs) are a cost-effective technique for diagnosing infections in the field (see the October 1988 issue of *ILRAD Reports).* Other technologies, based on the binding of complementary single strands of DNA (deoxyribonucleic acid), identify trypanosomes with unparalleled sensitivity (January *1991 ILRAD Reports).* The latter technologies have the further

advantage that DNA is more robust and stable than the proteins and antibodies used in the monoclonal antibody-based antigen-ELISAs. For the most part, however, the DNA-based technologies have been restricted to laboratory work because they require use of radioactive chemicals.

The standard polymerase chain reaction technique

The polymerase chain reaction technique is used to amplify specific regions of an organism's DNA to levels where the DNA fragment can be identified. The technique generally requires a pair of short pieces of single-stranded DNA composed of 20 nucleotide bases, called oligonucleotides, which are specifically synthesized for the reaction. Each paid of oligo-nucleotides is specific for the section of the genomic DNA of the organism being analysed. These oligonucleotides under particular conditions will bind to their complementary sequences in the genomic DNA and act as 'primers' that initiate copying of a specific region of the DNA of the organisms under study.

Two primers are used—one for each strand of the DNA double helix. The DNA strands of the double helix are copied, or synthesized, in opposite directions. Only a short region of the genome is amplified. Each round of amplification effectively doubles the copy number of the DNA region being amplified. After only 30 rounds of amplification, the copy number of the specific sequence increases over one million-fold. This amount of DNA is easily seen after staining with a DNA-binding reagent when the reaction products are separated by electrophoresis in a gel.

Several recent developments are now making these technologies attractive for field as well as laboratory use. These include an increasingly widespread use of the polymerase chain reaction (PCR) technique, the advent of new systems for detecting parasite DNA that require no radioactive labelling and are more sensitive and reliable than those used in the past, and increasing cost-effectiveness of the technologies. The DNA based technologies are thus likely in the future both to improve diagnosis of infections and to advance epidemiological studies.

The PCR technique is used to amplify a specific region of an organism's DNA to levels where the DNA fragment can be identified (see the box on this page and the October 1991 *ILRAD Reports).* Although powerful, PCR requires that a pair of short pieces of single-stranded DNA be synthesized specifically for each DNA sequence to be detected by subsequent amplification in the reaction. Moreover, to generate these primers, known as oligonucleotides, DNA of the relevant genes must first be sequenced. In addition, use of the PCR with a pair of primers for a specific DNA sequence does not produce different reaction fragments—seen in a gel as DNA bands of different sizes—and thus cannot distinguish isolates of the same species of trypanosome.

SCIENTISTS have now developed more refined systems for identifying organisms on the basis of stretches of their DNA. The breakthrough was made by two American groups that first identified closely related organisms using genetic 'fingerprints' produced by amplifying DNA of the organisms with arbitrary primers. This technique, soon dubbed 'RAPD' for 'random amplified polymorphic DNA' (the method is also, less euphoniously, known as AP, for 'arbitrary primer'-PCR), uses single primers of arbitrary sequence of only 10 nucleotides in length rather than the longer (20-nucleotide) pairs of primers for specific sequences used in the standard PCR method. Because of their shorter sequences, the arbitrary primers used in the RAPD technique will bind to many stretches of DNA in an organism's genome—that is, to the many sites that have a complementary, or nearly complementary, sequence of nucleotide bases.

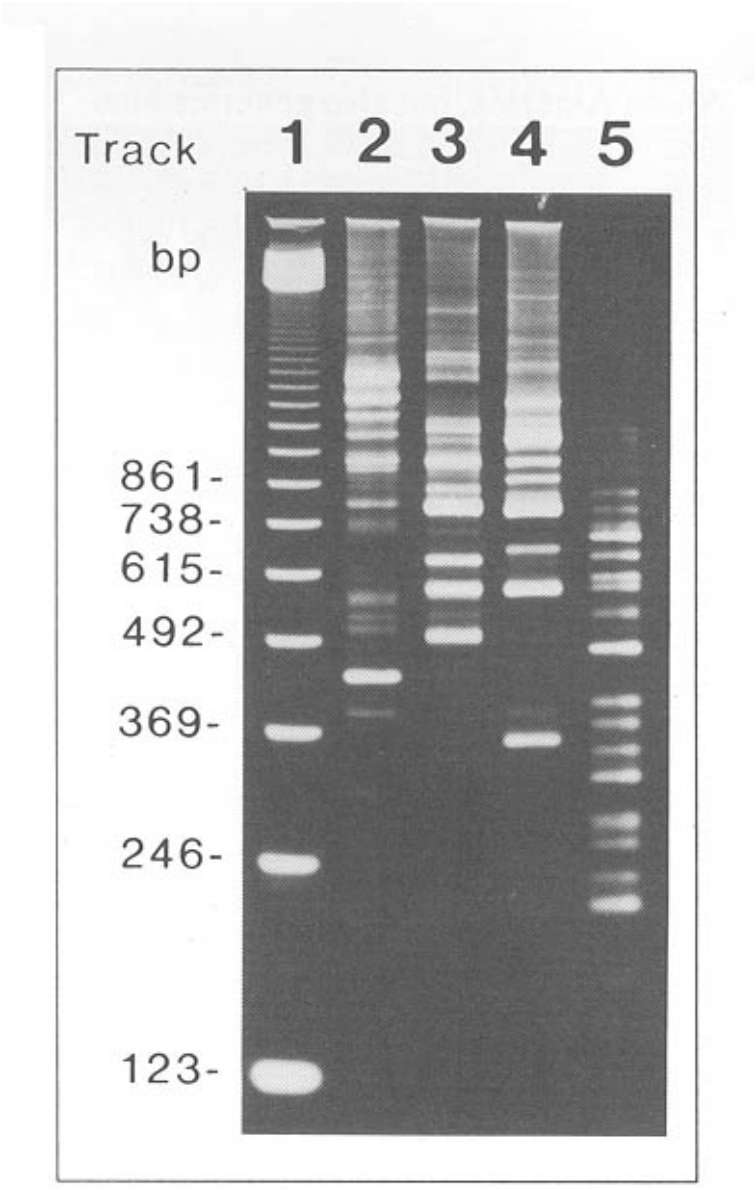
As organisms evolve, fragments of their DNA may be deleted or inserted or changed through genetic mutation. The arbitrary primers point up such differences among closely related populations by generating different-sized PCR products, which appear as different-sized bands on a gel, or by generating products with DNA from one population (bands) but no products with DNA from another population (no bands). The differences are easily demonstrated by running the DNA amplified from different organisms alongside each other in gels (see the figure on this page). The degree to which two organisms are related is indicated by the degree to which the fingerprint or pattern of bands produced in a RAPD reaction are similar.

The RAPD technique thus overcomes some limitations of the standard PCR reaction: it enables scientists to generate a fingerprint of the genome of an organism that can be used to identify the organism rapidly, even when only a few of the organisms are present in samples being analysed.

The main advantages of the RAPD method are that a single short primer is sufficient to differentiate many different organisms and that to produce the primer the experimenter needs no prior information about DNA sequences in the organism of interest. The technique can generate fingerprints of different populations of the same species, which can then be used to distinguish the populations from each other. Due to the complexity of trypanosomiases, this advantage is particularly important in detailed studies of trypanosome infections in the field. For example, individual parasite populations that are particularly virulent could be followed and their influence on the epidemiology of the disease in the field monitored.

SCIENTISTS at ILRAD have used the RAPD technique to determine differences among and within species of trypanosomes. The fingerprints produced from DNA of members of three trypanosome subgenera —*Nannomonas, Trypanozoon,* and *Duttonella*—have been examined after using different arbitrary 10-mer oligonucleotides to amplify DNA from these organisms. Of the primers tested, one designated ILO 525, with a nucleotide base sequence of 5' CGGACGTCGC 3', distinguished the three subgenera from each other and also produced identifiable fingerprints for the different parasite populations within each of the subgenera.

The different fingerprint patterns, or polymorphisms, generated using this primer allowed scientists easily to differentiate five previously identified subtypes of the *Nannomonas* group: *Trypanosoma simiae* and the Kilifi-, Tsavo-, savannah- and West African forest/riverine-types of *Trypanosoma congolense.* Although *T. simiae* is morphologically indistinguishable from other members of the *Nannomonas* subgenus, most of which are pathogenic in cattle, *T. simiae* is the only one of the subgroup to be particularly pathogenic in pigs.



The different DNA products of four Nannomonas isolates—Trypanosoma congolense *Kilifi (lane 2), forest/riverine (West African), (lane 3) and savannah (lane 4), and* Trypanosoma simiae *(lane 5)—generated using the RAPD method, separated by electrophoresis in a gel and stained with a dye that binds to the DNA and allows it to be visualized with ultraviolet light.*

RAPD fingerprints of *T. simiae* populations differed markedly from the fingerprints of the other members of the *Nannomonas* group. For all the *T. congolense* parasites analysed, oligonucleotide ILO 525 generated fingerprints that not only unequivocally identified the species but also revealed polymorphisms among the different isolates of the same subspecies.

Disadvantage of RAPDs

A disadvantage of using the RAPD technique to identify trypanosomes is that the parasites must be purified from host nucleate cells: any contamination by host DNA will alter the fingerprint patterns.

This separation is achieved by passing host blood samples over DEAE-cellulose columns. To be effective, this technique requires relatively that the host have high degrees of parasitaemia to provide sufficient numbers of parasites.

Modifications of this technique or new procedures for isolating trypanosomes need to be developed before the RAPD technique can reach its full potential in diagnosis and epidemiological studies of trypanosomiasis.

THE FINGERPRINTS produced using primer ILO 525 divided members of the *Trypanozoon* subgenus into three major groups: (1) *Trypanosoma brucei brucei and T. b. rhodesiense,* (2) *T. evansi* and (3) *T. b. gambiense.*

Group one comprised *T. b. brucei* populations from East and West Africa, which do not infect humans, and *T. b. rhodesiense,* which is found in several areas in Africa and does infect people. Although the fingerprints did not distinguish *T. b. brucei* from *T. b. rhodesiense,* the profiles in group one were sufficiently different to identify different isolates. The inability of the RAPD method to differentiate *T. b. brucei* and *T. b. rhodesiense* lends support to results of DNA hybridization and isoenzyme comparisons that suggest that these two subspecies are components of a single species group and that *T. b. brucei* can potentially infect people.

Group two comprised all isolates of *T. evansi,* which are morphologically similar to the other members of the *Trypanozoon subgenus* but are transmitted globally in tropical and subtropical regions not by tsetse but by other biting flies. Their distinction from other *Trypanozoon* isolates was indicated by the absence of one DNA band in the fingerprint and the presence of a larger, less intense band. Analysis of the sequences of the two DNA bands showed them to differ from each other. To date, the RAPD technique has not revealed qualitative differences among the *T. evansi* isolates. More oligonucleotides need to be tested to identify arbitrary primers that will reveal differences in the fingerprints of these *T. evansi* isolates.

Group three comprised *T. b. gambiense* isolates from West Africa. This organism causes human sleeping sickness. The RAPD technique**,** again carried out with ILO 525, generated two sorts of DNA fingerprints for the *T. b. gambiense* isolates tested. Both sorts of fingerprints lacked the two bands that distinguish the other two *Trypanozoon* groups. This division of *T. b. gambiense* into two types agrees with earlier subdivisions of isolates of this organism that were made on the basis of different variant surface glycoprotein genes possessed by the two types.

Application of the RAPD technique using *T. vivax* isolates of the *Duttonella* subgenus from South America and Africa has confirmed results of previous analyses and facilitated differentiation of isolates that in the past could be accomplished only by employing a combination of several relatively tedious techniques. Primer ILO 525 was again found to be the most useful in distinguishing these parasites. The RAPD method not only placed all non-Kenyan *T. vivax* into one group containing widely distributed isolates from Africa and South America, but also generated isolate-specific patterns that may, in the future, enable scientists to identify regions of the genome encoding genes responsible for the expression of different traits, such as resistance to drugs and an ability to infect rodents, to induce haemorrhagic disease and to be transmitted by tsetse.

The similarities observed between the West African and South American (Colombian) *T. vivax* populations supports the generally held hypothesis that the South American parasites are derived from tsetse-transmitted West African trypanosomes. The number of South American parasites sampled so far, however, is insufficient to determine the degree of heterogeneity among the South American isolates and whether this parasite was imported from Africa on one or on several occasions.

IN SUMMARY, use of the single oligonucleotide primer ILO 525 at ILRAD in the RAPD technique—rather than pairs of primers needed to identify each different parasite population in the standard PCR technique—has enabled scientists to differentiate the different subgenera, species and individual isolates of trypanosomes from Africa and South America. This advantage greatly simplifies and extends the possibilities in studies of the epidemiology of trypanosomiasis.

In the future, scientists may use this technique to identify genes expressing traits of importance. Like other PCR based methods, the RAPD method uses very small quantities of DNA (billionths of a gram) and therefore can be used to identify infecting organisms even when samples contain as few as a hundred parasites.

ILRAD Reports is published quarterly by the International Laboratory for Research on Animal Diseases (ILRAD) in Nairobi,. Kenya. The newsletter is written, designed and electronically laid out to camera-ready stage in-house using a personal computer and laser printer. The newsletter is printed in Nairobi by Majestic Printing Works. It has a circulation of 3 500 English and 500 French readers and is available free upon request. Material in the *Reports* may be reproduced without prior permission but we ask that credit be given and that two copies of the reprint be sent to ILRAD's Science Writer, Susan MacMillan: telephone + 254 (2) 632-311 telefax + 254 (2) 631-499 telex 22040 ILRAD. electronic mail: (BT Tymnet) CG1017

ILRAD was founded in 1973 to conduct research into better ways of controlling livestock diseases. The current primary goal of the Laboratory is to develop safe, effective and economical methods to control two parasitic diseases that severely constrain animal production in Africa:trypanosomiasis, transmitted to animals by the bite of a tsetse fly, and East Coast fever, a virulent form of theileriosis, transmitted to cattle by ticks. An international staff of about 50 scientists conducts basic research much of it aimed at the development of vaccines in the fields of biochemistry, cell biology, electron microscopy, epidemiology, genetics, immunology, molecular biology, pathology, parasitology and the socio economics of animal disease control

ILRAD is one of 18 international agricultural research centres sponsored by the Consultative Group on International Agricultural Research (CGIAR). The secretariat of the CGIAR is located in the World Bank headquarters in Washington D.C. The CGIAR is an informal umbrella organization of 40 national governments, international organizations and private foundations that together provide about US$300 million annually to the 18 centres for research., training and advisory services. The CGIAR aims to help farmers in developing countries increase their production of staple food crops livestock fish and trees in ways that improve the nutrition and well-being of low-income peoples and the management of natural resources.